JOURNAL OF VIROLOGY, Mar. 2004, p. 2277–2287 Vol. 78, No. 5
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.5.2277–2287.2004

Role for CCR5Δ32 Protein in Resistance to R5, R5X4, and X4 Human Immunodeficiency Virus Type 1 in Primary CD4$^+$ Cells

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Received 30 June 2003/Accepted 11 November 2003

CCR5Δ32 **is a loss-of-function mutation that abolishes cell surface expression of the human immunodeficiency virus (HIV) coreceptor CCR5 and provides genetic resistance to HIV infection and disease progression. Since CXCR4 and other HIV coreceptors also exist, we hypothesized that *CCR5Δ32*-mediated resistance may be due not only to the loss of CCR5 function but also to a gain-of-function mechanism, specifically the active inhibition of alternative coreceptors by the mutant CCR5Δ32 protein. Here we demonstrate that efficient expression of the CCR5Δ32 protein in primary CD4$^+$ cells by use of a recombinant adenovirus (Ad5/Δ32) was able to down-regulate surface expression of both wild-type CCR5 and CXCR4 and to confer broad resistance to R5, R5X4, and X4 HIV type 1 (HIV-1). This may be important clinically, since we found that CD4$^+$ cells purified from peripheral blood mononuclear cells of individuals who were homozygous for *CCR5Δ32*, which expressed the mutant protein endogenously, consistently expressed lower levels of CXCR4 and showed less susceptibility to X4 HIV-1 isolates than cells from individuals lacking the mutation. Moreover, CD4$^+$ cells from individuals who were homozygous for *CCR5Δ32* expressed the mutant protein in five of five HIV-exposed, uninfected donors tested but not in either of two HIV-infected donors tested. The mechanism of inhibition may involve direct scavenging, since we were able to observe a direct interaction of CCR5 and CXCR4 with CCR5Δ32, both by genetic criteria using the yeast two-hybrid system and by biochemical criteria using the coimmunoprecipitation of heterodimers. Thus, these results suggest that at least two distinct mechanisms may account for genetic resistance to HIV conferred by *CCR5Δ32*: the loss of wild-type CCR5 surface expression and the generation of CCR5Δ32 protein, which functions as a scavenger of both CCR5 and CXCR4.**

Human immunodeficiency virus type 1 (HIV-1) infection is mediated by binding of the viral envelope protein gp120 to two proteins on the surfaces of target cells, namely CD4 and a coreceptor. The coreceptor is almost always a chemokine receptor, typically either CCR5 or CXCR4 (reviewed in reference 1). HIV-1 strains can be divided into three major groups based on their coreceptor specificity as follows: R5 (CCR5-tropic), X4 (CXCR4-tropic), and R5X4 (able to use either CCR5 or CXCR4).

The importance of CCR5 in HIV infection and pathogenesis was revealed by the discovery of the *CCR5Δ32* allele, a 32-bp deletion in the portion of the human *CCR5* open reading frame (ORF) that encodes the second extracellular loop between transmembrane domains four and five of the seven-transmembrane domain architecture (16, 21, 25, 37, 50). *CCR5Δ32* encodes a truncated protein, designated Δ32 in this study, that is not detected on the cell surface and therefore is not functional as a coreceptor (16, 21, 25, 37, 50). The CCR5Δ32 mutant protein has 215 amino acids and an apparent molecular mass of 30 kDa, while wild-type (wt) CCR5 has 352 amino acids and an apparent molecular mass of 46 kDa.

CCR5Δ32 is common among Caucasians (~10% allele frequency in North America) but is absent or present at a very low frequency in native African and Asian populations (16, 25, 37, 50). According to the Hardy-Weinberg test, it has no effect on reproductive fitness; moreover, the homozygotes that have been evaluated appear healthy. Mice lacking CCR5 have been prepared by gene targeting and they too appear healthy (48). In rare cases, *CCR5Δ32* homozygosity has been associated with HIV-1 infection (reviewed in references 26 and 30), but in these cases the mechanism of infection has not been defined. Heterozygous individuals (+/−) are not protected against infection, but once they are infected, the progression to AIDS is slightly delayed (16, 25, 37, 50), indicating that partial resistance can occur in the presence of a single copy of *CCR5Δ32*. An analysis of T cells from heterozygous (+/−) individuals revealed markedly reduced CCR5 expression compared to cells from homozygous (+/+) individuals (42, 45).

In vitro expression studies have demonstrated that mutant CCR5Δ32 protein is retained in the endoplasmic reticulum and exerts a transdominant negative (TDN) effect on wt CCR5, impairing its transport to the cell surface (8, 13). These findings suggested that in addition to reduced gene dosage, CCR5-Δ32 heterodimerization may be a molecular mechanism for slower progression to AIDS in *CCR5Δ32* heterozygotes. However, the extent to which this occurs in primary CD4$^+$ cell targets of HIV-1 has not been analyzed, nor has it been determined whether the mutant protein affects CXCR4 expression and function. Here we address both of these issues. Our results suggest that resistance to HIV-1 infection in *CCR5Δ32* ho-

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mozygotes may result from both the genetic loss of CCR5 on the cell surface and the active down-regulation of CXCR4 expression by the mutant CCR5Δ32 protein. We also demonstrate for the first time that the expression of recombinant CCR5Δ32 protein in primary $CD4^+$ cells confers broad protection against R5, R5X4, and X4 HIV-1 infection.

MATERIALS AND METHODS

Cells and viruses. HeLa cells purchased from the American Type Culture Collection (Rockville, Md.) were cultured in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, Md.) containing 10% fetal bovine serum (FBS) (HyClone, Logan, Utah), 2 mM L-glutamine, and antibiotics. Recombinant viruses vCB-21R (pT7-*LacZ*), vTF7-3 (T7 polymerase), vCB-16 (Unc), vCB-43 (BaL), and vCB-41 (LAV) have been previously described (3). The HIV-1 isolates IIIB, Ba-L, and 89.6 were obtained from the NIH AIDS Reagent Program, Rockville, Md.

Recombinant vaccinia virus encoding CCR5 was obtained from Chris Broder (Uniformed Services University of the Health Sciences, Bethesda, Md.). Construction of a recombinant vaccinia virus encoding the CCR5Δ32 protein was performed as previously described (12). Briefly, the cDNA fragment encoding the CCR5Δ32 protein was inserted into p1107 under the control of the vaccinia virus 7.5 promoter and was transfected into thymidine kinase-negative cells that were infected with the Western Reserve wt strain of vaccinia virus. The Western Reserve virus does not encode any foreign proteins and was used as a negative vector control. Positive plaques were identified by dot blot hybridization using ^{32}P-labeled CCR5Δ32 cDNA. After three cycles of plaque purification on thymidine kinase-negative cells, virus stocks were prepared by infection of HeLa cells and were frozen at −70°C.

Peripheral blood mononuclear cells (PBMCs) from healthy anonymous blood donors who were homozygous for *CCR5Δ32* were collected at the Division of Transfusion Medicine, Warren Grant Magnuson Clinical Center, according to an NIH institutional review board approved protocol. A detailed description and an analysis of these samples have been previously described (50). PBMCs from all donors were either used as a total population or to purify the $CD4^+$ fraction by positive selection using microbeads coated with antibodies against CD4 according to the instructions provided by the manufacturer (Miltenyi Biotec, Auburn, Calif.). Briefly, the PBMCs were magnetically labeled with CD4 microbeads and the cell suspension was loaded onto a column that had been placed in the magnetic field of a magnetic cell-sorting separator. The magnetically labeled $CD4^+$ cells retained in the column were separated from the magnetic beads by removal of the column from the separator (removes the magnetic field) and placement of the column on a suitable tube. The $CD4^+$ cells were eluted from the column by use of a plunger.

PBMCs or purified $CD4^+$ cells were activated with phytohemagglutinin (PHA) (10 μg/ml) (Sigma Chemicals, St. Louis, Mo.) and 100 U of recombinant human interleukin-2 (rIL-2) (NIH AIDS Reagent Program)/ml for 3 days before use. PBMCs from two HIV-infected (−/−) individuals were obtained from H. Naif, Sydney, Australia, and H. Sheppard, San Francisco, Calif. The genotype of these samples was confirmed by reverse transcription (RT)-PCR. RT-PCR was performed on the total RNA (0.5 μg) isolated from uninfected and infected (−/−) PBMCs. Forward and reverse oligonucleotide primers for amplification were 5′-TGTGAAGCAAATCGCAGCCC-3′ and 5′ ATGGTGAAGATAAGA GCCTCACAGCC-3′, respectively. The primers were designed to amplify a 616-bp CCR5Δ32 fragment and a 648-bp CCR5 fragment.

Antisera against the CCR5Δ32 carboxy terminus. To obtain antisera specific for the CCR5Δ32 protein, rabbits were immunized with a peptide corresponding to the carboxy terminus of the protein (IKDSHLGAGPAAACHGHLLLGN-PKNSASVSK) conjugated to keyhole limpet hemocyanin. The carboxy-terminal region was chosen because this region has no shared amino acid homology with CCR5, and antibodies against this domain are specific for the CCR5Δ32 protein. The immunoglobulin G fraction was purified from crude antisera by using protein G-Sepharose, and further purification was performed by using a CCR5Δ32-specific peptide affinity column. The peak fraction containing affinity-purified antibodies was stored at −70°C until use.

FACS analysis. Cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (supplemented with 0.5% FBS and 0.02% sodium azide), resuspended in 100 μl of FACS buffer at 10^7/ml, and incubated with a 1:200 dilution of monoclonal antibodies (MAbs) raised against the different coreceptors at 4°C for 30 min. All MAbs and matched isotype controls were purchased from Pharmingen Inc., San Diego, Calif. Cells were then washed twice, resuspended in 100 μl of ice-cold FACS buffer in the presence of phycoerythrin (PE)-conjugated anti-mouse immunoglobulin G, and incubated at 4°C for 30 min. Finally, cells were washed twice, resuspended in 500-μl of ice-cold FACS buffer, and analyzed in a FACScan cytometer (Becton Dickinson).

Fresh PBMCs from 10 different donors were isolated by Ficoll centrifugation. Three-color flow cytometry was performed on unstimulated PBMCs or on cells stimulated with either PHA plus IL-2 or αCD3 antibody plus IL-2 for 3 days. A PE-conjugated MAb against CXCR4 (12G5), a fluorescein isothiocyanate-conjugated MAb against CCR5 (2D7), a cychrome-conjugated MAb against CD4 (RPA-T4), and a cychrome-conjugated CD3 MAb (HIT3Aa) were obtained from Pharmingen. Staining was performed by incubating 10^6 PBMCs in phosphate-buffered saline (PBS) containing 1% bovine serum albumin with the respective MAb(s) alone or in combination depending on the gate used (i.e., either CD3 or CD4 gated). Incubations were done for 30 min at 4°C and reaction mixtures were washed three times before analysis in a FACScan apparatus (Becton Dickinson).

Construction of recombinant vectors encoding CCR5 or CCR5Δ32. DNA fragments encoding either CCR5 or CCR5Δ32 were subcloned into pMCV-SV24 under control of the major late promoter of adenovirus type 2. The plasmid construct and method used to generate recombinant adenoviruses encoding measles virus hemagglutinin (MVHA) have been previously described (2). We have used this method to generate recombinant adenoviruses carrying several genes of measles virus (2, 5, 6). Briefly, CCR5 or CCR5Δ32 cDNA was inserted by homologous recombination into the early region of the adenovirus type 5 (Ad5) genome, replacing the E1 region (reviewed in references 10 and 18). The resulting E1-deficient viruses are defective for replication and are propagated to high titers in human 293 complementation cells by providing the missing E1 gene products in *trans* (19). Efficient expression of CCR5Δ32 or CCR5 by Ad5/Δ32 or Ad5/CCR5 is accomplished by infecting primary cells at a high multiplicity of infection, since these recombinant Ad5 vectors are replication defective in cells other than 293 cells.

HIV-1 Env-mediated fusion using Ad5/CCR5-infected target cells. The basic features of the fusion assay were developed by using the HIV-1 Env-CD4 interaction of two different populations of cells, with one expressing CD4 and the other expressing the HIV-1 envelope glycoprotein (Env) (29). Target cells (purified $CD4^+$ cells) were coinfected with Ad5/Δ32, Ad5/CCR5, Ad5 vector, or Ad5/MVHA and Ad5Pol3. Ad5Pol3 was obtained from Frank Graham, McMaster University, Hamilton, Ontario, Canada. Ad5Pol3 is a recombinant adenovirus that encodes T7 RNA polymerase. HeLa cells coinfected with vCB-21R (LacZ under control of the T7 promoter) and one of the HIV-1 Env proteins served as effector cells. After mixing of the effector and target cell populations and incubation at 37°C for 2.5 h, the fusion specificity was measured by β-galactosidase (β-Gal) production in a colorimetric lysate assay. In experiments shown in Fig. 6, 293-CD4 cells were used to analyze CCR5Δ32 protein interactions and the protein's effect on Env-mediated cell fusion. 293-CD4 cells were coinfected with either Ad5/CCR5 plus Ad5/Δ32 or Ad5CCR5 plus Ad5 and Ad5Pol3 and were challenged with HeLa cells expressing HIV-1 Env and LacZ under control of the T7 promoter.

Western blot analysis. For Western blotting, cell lines or primary $CD4^+$ cells purified from $CCR5^{+/+}$ PBMCs were infected with Ad5/Δ32, and the expression of CCR5Δ32 was monitored by immunoblotting. For CCR5Δ32 protein detection in $CCR5\Delta32^{-/-}$ individuals, primary $CD4^+$ cells were purified from $CCR5\Delta32^{-/-}$ PBMCs. Cell lysates were prepared, fractionated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (PAGE), and immunoblotted onto polyvinylidene difluoride membranes (Millipore). After blocking, membranes were reacted with polyclonal antibodies directed against the N terminus of CCR5 (5) or with affinity-purified CCR5Δ32-specific antibodies at a 1/100 dilution, washed, and incubated with horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected by the addition of a substrate.

Analysis of CCR5Δ32 protein interactions. The yeast Matchmaker two-hybrid system 3 (GAL4-based) was utilized to study protein-protein interactions as suggested by the manufacturer (Clontech). The system provides a transcriptional assay for the detection of protein interactions in vivo in the yeast *Saccharomyces cerevisiae*. The CCR5Δ32 ORF was expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while CCR5 or CXCR4 was expressed as a fusion to the GAL4 activation domain (AD). The DNA-BD is amino acids 1 to 147 of the yeast GAL4 protein, which binds to the GAL upstream activation sequence upstream of the reporter genes. If CCR5 or CXCR4 interacted with CCR5Δ32, AD amino acids 768 to 881 act as a transcriptional activator to drive expression of the reporter genes. Strain AH109, which uses the reporters Ade2, HIS3, and LacZ under the control of distinct GAL4 upstream activity sequences, was used to study coreceptor-CCR5Δ32 interactions. At medium stringency, HIS3 was used as a reporter gene, with 10 mM 3-amino-1,2,4-triazole (3-AT) as a competitive inhibitor.

Complex formation, or heterodimerization, between CCR5Δ32 and either CCR5 or CXCR4 was analyzed in 293-CD4 cells that had been coinfected with Ad5Pol3 and either Ad5/CCR5 plus Ad5/Δ32 or Ad5/CCR5 plus Ad5. At the appropriate time after infection, a portion of infected cells was used in a cell fusion assay as described above, and the other portion was lysed in RIPA buffer (0.5% Triton X-100, 250 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.2 mM phenylmethylsulfonyl fluoride) containing 0.1% bovine serum albumin, rabbit polyclonal anti-CCR5Δ32 serum, and protein A-Sepharose. For resolution of the protein complexes, the precipitated proteins (complexed to protein A-Sepharose) were washed three times in RIPA buffer and resuspended in perfluorooctanoic acid (PFO) loading buffer (50 mM Tris base [pH 8.0], 4% [wt/vol] NaPFO, 10% glycerol, and 0.005% bromophenol blue). Samples were fractionated in a freshly poured 10% Tris-glycine-acrylamide gel without sodium dodecyl sulfate. The running buffer contained 25 mM Tris, 192 mM glycine, and 0.5% PFO. It was previously demonstrated that the detergent PFO protects interactions within protein oligomers (34). The fractionated proteins were transferred onto a polyvinylidene difluoride membrane by blotting (Millipore). After blotting, the membrane was treated with 5% skim milk powder in PBS for 2 h, incubated with anti-CCR5Δ32 antibody for 16 h at 4°C, and washed with PBS containing 0.1% Triton X-100. Bound antibodies were detected by incubation with horseradish peroxidase-conjugated secondary antibodies and the addition of a substrate after washing. The membrane was stripped and used to react with an anti-CCR5 MAb (CTC-6; Protein Design Labs). After detection, the membrane was stripped again and reacted with antibodies specific to CXCR4 (17).

HIV-1 infection of wt primary CD4$^+$ cells transduced with Ad5/Δ32. PHA-plus-IL-2-activated CD4$^+$ cells isolated from an HIV-seronegative (+/+) donor were infected with either Ad5/Δ32 or Ad5/CCR5 at a multiplicity of infection of 50 PFU/cell. Infected cells were incubated for 2 days to allow the expression of recombinant proteins (CCR5 or CCR5Δ32 protein) and then were infected with either IIIB (X4), Ba-L (R5), or 89.6 (R5X4) HIV-1. The virus was adsorbed for 2 h, and cells were then washed three times with PBS and maintained in RPMI 1640 supplemented with 10% FBS, PHA, and IL-2. The culture fluid (50 μl) was harvested after cell resuspension every 3 days and was replaced with fresh medium. The amount of p24 antigen in the cell-containing supernatants was measured by using an enzyme-linked immunosorbent assay kit purchased from DuPont (Wilmington, Del.).

RESULTS

Reduced susceptibility to X4 HIV-1 infection of primary CD4$^+$ cells isolated from *CCR5Δ32* homozygous individuals. A biological activity of CCR5Δ32 protein has been described as downmodulating CCR5 at the cell surface in a TDN manner (8, 13). The recent isolation of X4 (27) and R5X4 (28) from infected (−/−) individuals suggested that viral transmission occurred through CXCR4. However, why CXCR4 is rarely used in uninfected (−/−) individuals remains an unresolved question. To investigate this issue, we first compared the susceptibility to X4 HIV-1 infection of CD4$^+$ cells purified from four HIV-uninfected *CCR5Δ32* homozygotes (−/−) and from individuals carrying the homozygous wt *CCR5* allele (+/+). The genotypes of these individuals were confirmed by RT-PCR amplification of the fragment spanning the 32-bp deletion (Fig. 1A). CD4$^+$ cells purified from −/− or +/+ individuals will be referred to as −/− or +/+ CD4$^+$ cells. The +/+ CD4$^+$ cells supported high levels of both X4 and R5 HIV infection. The −/− CD4$^+$ cells did not support R5 HIV infection, as expected, but unexpectedly were also markedly deficient compared to +/+ cells in the ability to support X4 HIV infection (Fig. 1B). This correlated with a marked reduction of CXCR4 expression on the surfaces of −/− versus +/+ cells by FACS, whereas CD4 levels were similar (Fig. 1C and D). We hypothesized that *CCR5Δ32* exerts a TDN effect on CXCR4 expression through expression of the CCR5Δ32 protein.

Stimulation of PBMCs reduces the percentage of CCR5$^+$ CXCR4$^+$ double-positive cells. To exert a TDN effect on CXCR4, the mutant CCR5Δ32 protein must be coexpressed in CXCR4$^+$ cells. Previous studies reported a low percentage of CCR5$^+$ CXCR4$^+$ cells in CD3-gated, PHA-plus-IL-2-stimulated T lymphocytes (11). To readdress this issue, we performed three-color immunofluorescence flow cytometry on PBMCs from 10 healthy donors and found that the percentage of cells coexpressing CCR5 and CXCR4 is four- to fivefold higher in unstimulated PBMCs than in PHA and IL-2 blasts (Fig. 2 and Table 1). The percentage of double-positive cells depended on the cell population gated and the method of stimulation. For all donors tested, the percentage of double positives in CD3-gated cells was lower than in the CD4-gated cell population (Fig. 2 and Table 1). The reduction was donor dependent and also depended on the cell activation method. For seven donors, PHA plus IL-2 stimulation resulted in the redistribution of coreceptor expression, causing a dramatic reduction in the percentage of double-positive cells. All 10 donors tested showed a dramatic reduction in double-positive cells (CD3 gated) upon anti-CD3 plus IL-2 stimulation. The results demonstrate that the percentage of CCR5$^+$ CXCR4$^+$ cells was consistently higher in unstimulated CD4-gated cells (34.33 to 76.5%) than in CD3-gated cells (16 to 43%). Table 1 also shows the percentages of CD4$^+$ CXCR4$^+$ cells that coexpress CCR5. These values may represent the percentages of CD4$^+$ CXCR4$^+$ cells that could potentially be affected by

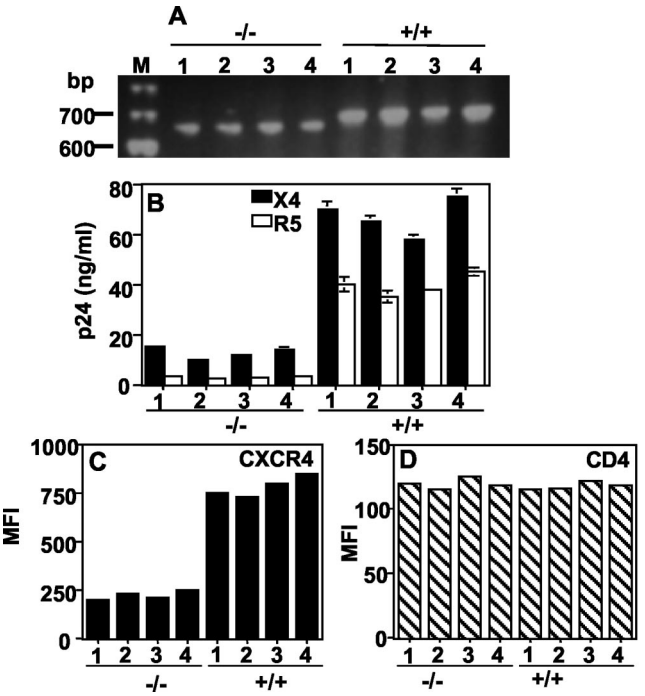

FIG. 1. CD4$^+$ cells from individuals homozygous for *CCR5Δ32* (−/−) are resistant to infection with both X4 and R5 HIV. (A) The genotypes of the PBMC samples were verified by RT-PCR amplification of a fragment that spans the 32-bp deletion. CD4$^+$ cells purified from four −/− and four +/+ individuals were stimulated with PHA plus IL-2 for 3 days and were used in an HIV-1 infection assay (B) or for FACS analysis of CXCR4 (C) or CD4 (D). The p24 values in panel B represent amounts produced at day 9 postinfection. The results shown are from a single experiment representative of four independent experiments. MFI, mean fluorescence intensity; M, DNA size marker.

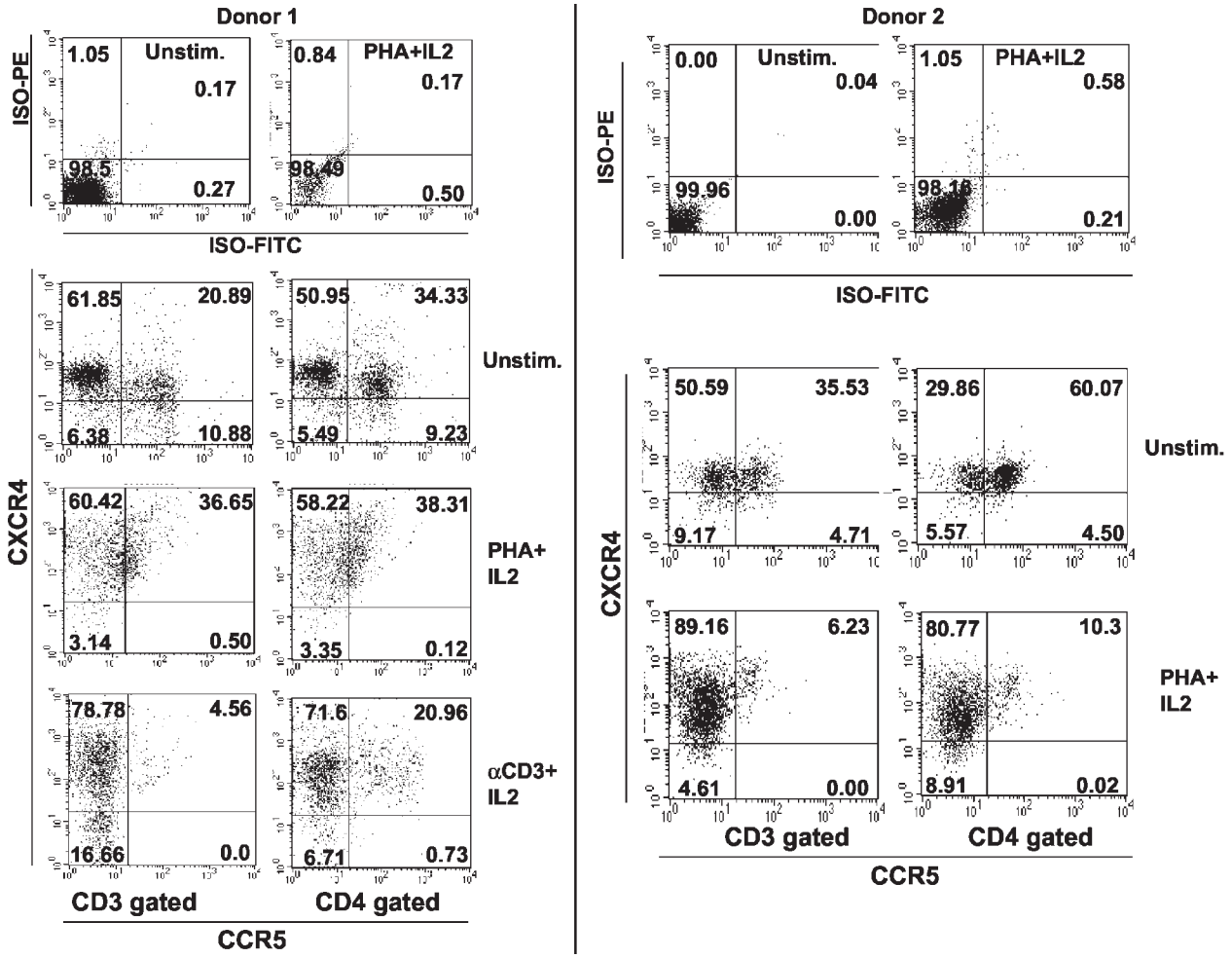

FIG. 2. CCR5$^+$ CXCR4$^+$ double-positive cells in unstimulated and stimulated PBMCs. Three-color flow cytometry of freshly isolated PBMCs was performed on either unstimulated cells or cells stimulated with PHA plus IL-2 or αCD3 antibody plus IL-2. PBMCs were gated according to forward and side scatter and with either CD3-cychrome (HIT3Aa) or CD4-cychrome (RPA-T4) staining. The two-dimensional plots show expression of CCR5 (fluorescein isothiocyanate conjugate; 2D7 MAb) versus CXCR4 (PE conjugate; 12G5 MAb). Percentages of cells that are positive in the respective quadrants are indicated. Results for the respective isotype control antibodies are shown in the top two plots for each donor. The cell treatment is indicated to the right of each pair of plots, and gating is indicated at the bottom of each column of plots. The figure shows primary data from two different individuals and Table 1 summarizes the results from these two donors and eight others.

coexpression of the CCR5Δ32 protein in −/− individuals. The results of this analysis demonstrate that the percentage of double-positive cells (CXCR4$^+$ CCR5$^+$) is higher than was previously thought and may explain the three- to fourfold drop in sensitivity to X4 virus infection shown in Fig. 1B.

Endogenous expression of the truncated CCR5Δ32 protein. The genotypes of PBMCs isolated from −/− and +/+ individuals were confirmed by RT-PCR analysis using primers that span the 32-bp deletion (Fig. 3A). To analyze the potential protective effects of the CCR5Δ32 protein, we examined whether the truncated protein is expressed in CD4$^+$ cells purified from −/− or +/+ PBMCs by using a polyclonal antiserum raised against the frame-shifted 31 amino acid residues found in CCR5Δ32 but not in CCR5. The analysis revealed a high level of expression of the CCR5Δ32 protein, which appeared as a 30-kDa band on a Western blot of CD4$^+$ cells purified from PBMCs isolated from five different −/− individuals (Fig. 3B and C). The mutant CCR5Δ32 protein was also detected by using polyclonal antibodies against the N-terminal region of CCR5 (common to CCR5 and CCR5Δ32) (4) (Fig. 3C). Interestingly, the CCR5Δ32 protein was not significantly detected in CD4$^+$ cells purified from two different unrelated infected −/− individuals (Fig. 3B). As expected, we were un-

TABLE 1. Distribution of cells expressing both CCR5 and CXCR4 in resting and activated PBMCs[a]

Treatment	Mean % (range) of indicated cells in PBMC subset		
	CCR5$^+$ CXCR4$^+$		CCR5$^+$
	CD3 gated	CD4 gated	CD4$^+$ CXCR4$^+$ gated
Unstimulated	28.8 (16–43)	67.1 (34.33–76.5)	71.8 (40.26–81.25)
PHA plus IL-2	14.8 (5–36.65)	20.4 (10.3–38.31)	22.6 (11.3–39.85)
Anti-CD3 plus IL-2	5.6 (3–9.4)	14.3 (10.6–21.4)	15.8 (11.4–23.9)

[a] Ten PBMC samples were used in a three-color staining procedure to examine the percentages of CCR5$^+$ CXCR4$^+$ cells in CD3- versus CD4-gated cells. The races and identities of volunteers are unknown.

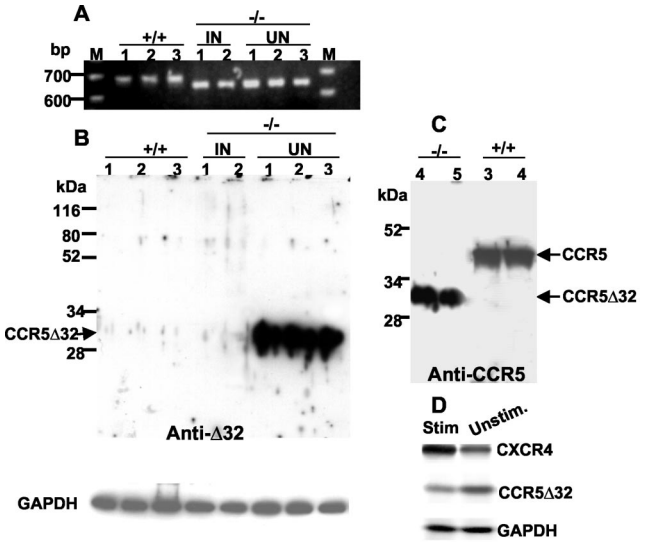

FIG. 3. Detection of endogenous CCR5Δ32 protein in unstimulated primary $CD4^+$ cells from CCR5Δ32 homozygotes. (A) Genotypes were confirmed by RT-PCR amplification of the appropriate fragment of CCR5 (648 bp) or CCR5Δ32 (616 bp) from purified $CD4^+$ cells from −/− and +/+ individuals. −/−, the genotype of individuals homozygous for the CCR5Δ32 allele; +/+, individuals homozygous for the wt CCR5 allele. The $CD4^+$ cells were purified from five HIV-uninfected (−/−) donors (identified as UN 1, 2, and 3 in panel B and 4 and 5 in panel C), two different HIV-infected (−/−) individuals (labeled IN 1 and 2), and three HIV-uninfected individuals lacking CCR5Δ32. The CCR5Δ32 protein was detected by using anti-CCR5Δ32 (anti-Δ32), a polyclonal rabbit antiserum directed against the novel frame-shifted amino acids that specifically recognizes the mutant protein (B), or antibodies against the common N terminus of CCR5 and CCR5Δ32 proteins (C). Probing similar blots with preimmune serum did not show any protein bands specific for the CCR5Δ32 protein (not shown). (D) Up-regulation of CXCR4 and down-regulation of CCR5Δ32 proteins upon PHA plus IL-2 stimulation of $CD4^+$ cells (Stim). Unstim., unstimulated. The numbers at the left in panel C indicate the positions of protein standards. kDa, kilodaltons; M, DNA size marker.

able to detect the protein in cells from any of the +/+ subjects tested (Fig. 3B and C).

To examine the effect of cell activation on protein expression, we compared the levels of the CCR5Δ32 protein made in PHA-plus-IL-2-stimulated cells to those in unstimulated $CD4^+$ cells. The results indicated down-regulation of CCR5Δ32 expression and up-regulation of CXCR4 expression upon stimulation (Fig. 3D).

Construction of recombinant adenoviruses encoding either CCR5 or CCR5Δ32. For analysis of the apparent TDN effect of the CCR5Δ32 protein in more detail, the cDNA fragment encoding either CCR5 or CCR5Δ32 protein was inserted into the genome of Ad5 (Fig. 4A). We used this expression system because Ad5 viruses are replication defective in PBMCs and do not cause the cytopathic effect associated with the wt virus and because primary cells infected with these vectors can be infected with HIV-1 and monitored for p24 production. To demonstrate that these vectors can deliver the CCR5 or CCR5Δ32 gene into most of the infected cells, we used a similar Ad5/GFP vector encoding green fluorescent protein (GFP). We used 293 cells as a target for either infection with Ad5/GFP (10 PFU/cell) or transfection with a plasmid carrying GFP under cytomegalovirus promoter. The results demonstrated that infection resulted in efficient GFP expression in most infected cells, whereas only 10% of the transfected cells expressed GFP (data not shown).

We found that the CCR5 protein encoded by Ad5/CCR5 is a functional receptor in terms of its coreceptor activity, as demonstrated by cell fusion assays. Immunoblot analysis of cellular lysates prepared from Ad5/Δ32-infected 293 cells revealed expression of the 30-kDa CCR5Δ32 protein, as detected with either CCR5Δ32-specific antibodies (Fig. 4B) or CCR5 antibodies directed against the common N terminus (Fig. 4C). Protein bands above the 34-kDa marker band were sometimes detected in CCR5Δ32-expressing 293 cells (Fig. 4B). These protein species could represent complexes between CCR5Δ32 molecules and cellular proteins or oligomers of the CCR5Δ32 protein itself. In order to determine that our adenovirus vector does not result in the overexpression of CCR5Δ32 protein, we demonstrated that the levels of Ad5-encoded CCR5Δ32 are comparable to those endogenously made in $CD4^+$ cells purified from −/− PBMCs (Fig. 4D). As expected, uninfected $CD4^+$ cells or cells infected with Ad5/CCR5 or Ad5 did not show any protein band corresponding to the CCR5Δ32 protein.

***trans*-downmodulation and impairment of HIV coreceptor activity.** For examination of the TDN effect of Ad5-encoded CCR5Δ32 protein, $CD4^+$ cells from healthy donors (wt CCR5) were infected with wt Ad5, Ad5/Δ32, or Ad5/CCR5 and stained with MAbs against CCR5, CXCR4, CXCR2, or CD4. FACS analysis demonstrated that primary $CD4^+$ cells expressing recombinant CCR5Δ32 protein, but not any other protein, showed specific downmodulation of CCR5 and CXCR4 (75 to 80% reduction) (Fig. 5A). Interestingly, expression of the CCR5Δ32 protein had no significant effect on cell surface CD4 or CXCR2, indicating the specificity of the downmodulation effect (Fig. 5A). In other experiments, we found that expression of the CCR5Δ32 protein did not affect CCR4 or CCR2 expression (data not shown). These downmodulation results were confirmed by using $CD4^+$ cells purified from PBMCs from 10 different donors.

For analysis of the effect of CCR5Δ32-induced downmodulation on CCR5 coreceptor activity, the same $CD4^+$ cells used for Fig. 5A were mixed with HeLa cells expressing either R5 or X4 HIV-1 Env. The extent of Env-mediated fusion was measured by a reporter gene activation assay (β-Gal production). The results indicated that CCR5Δ32 expression impaired the ability of R5 and X4 Env proteins to fuse with $CD4^+$ cells (Fig. 5B). As expected, R5 and X4 Env-mediated fusion was not reduced by the expression of Ad5 proteins (Ad5), CCR5, or MVHA. To exclude the possibility that the TDN effect was due to the Ad5 vector system, we repeated the analysis with a recombinant vaccinia virus vector encoding the CCR5Δ32 protein. The results confirmed those obtained with the Ad5 system (Fig. 5C and D). In contrast, the vaccinia virus-encoded CCR5Δ32 protein had no effect on fusion mediated by human T-cell leukemia virus type 1 Env, from a closely related retrovirus that recognizes a wide range of mammalian cell targets (41) (Fig. 5E). Thus, we were able to demonstrate the specificity of the TDN CCR5Δ32 activity against CCR5 and CXCR4 in two different vector systems.

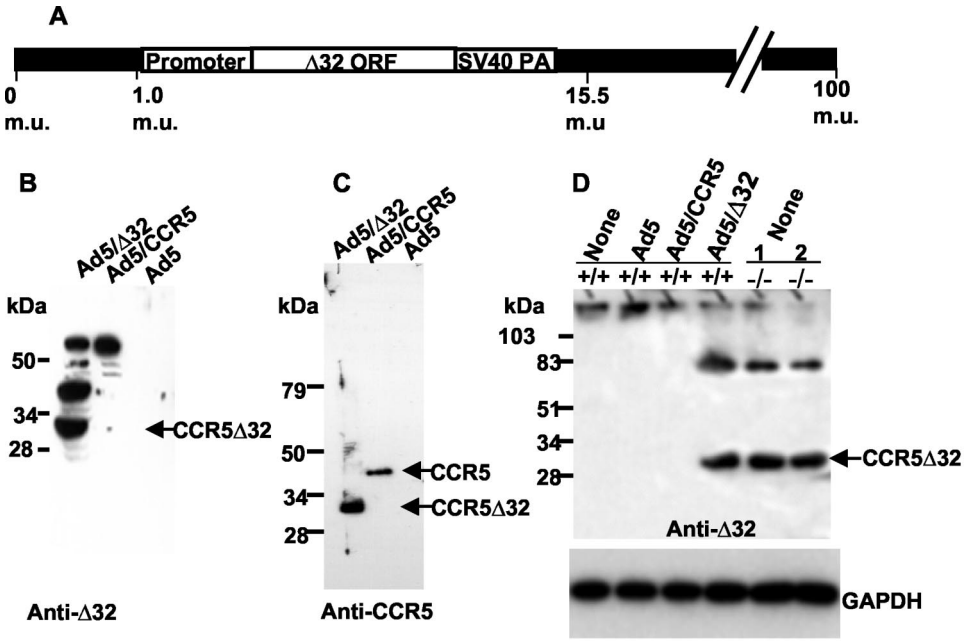

FIG. 4. Expression of CCR5Δ32 protein in 293 cells. (A) Structural map of a recombinant adenovirus (Ad5) encoding the Δ32 ORF. The CCR5Δ32 ORF, cloned from an HIV-seronegative CCR5Δ32 homozygote (−/−), is under the control of the major late promoter of adenovirus type 2 and the simian virus 40 poly(A) 3′ (SV40 PA) processing signal. The adenovirus genome is represented as 100 map units (m.u.), with 365 bp/m.u. (B and C) Detection of Ad5-encoded CCR5Δ32 protein in infected 293 cells. Ad5-encoded CCR5Δ32 protein was detected by using anti-CCR5Δ32 antisera (anti-Δ32) directed against the frame-shifted domain of CCR5Δ32 (B) or antisera directed against the CCR5 N terminus (anti-CCR5) (C). Note that bands above the 34-kDa marker band are frequently detected with anti-CCR5Δ32 but not with anti-CCR5 antibodies. (D) Comparison of CCR5Δ32 protein levels made in Ad5/Δ32-infected $CD4^+$ cells with those endogenously made in −/− $CD4^+$ cells. The blot was reprobed with antibodies against GAPDH to control for gel loading. PBMC samples from individuals homozygous for the CCR5Δ32 allele are referred to as −/−, and those from individuals homozygous for the wt CCR5 allele are referred to as +/+. Samples labeled as 1 and 2 are the same as samples 1 and 2 used in other figures. The numbers at the left indicate the positions of protein standards. kDa, kilodaltons.

Analysis of CCR5Δ32 interaction with CCR5 and CXCR4. The yeast two-hybrid system has been used as a powerful genetic tool to rapidly select previously uncharacterized proteins and to identify novel components of signaling pathways. The expression of functional CXCR4 in yeast has recently been described (35). The yeast two-hybrid system has been previously used to demonstrate CCR5Δ32 protein interaction with wt CCR5 (8). To control for nonspecific interactions, we used either empty vectors (Table 2, rows 1, 3, 4, and 5) or a vector that expressed a nonrelevant protein (pGADT-7-MVHA). A positive interaction was detected upon cotransformation with pGBKT7-53 plus pGBKT7-TAg (positive control), pGBKT-7-CCR5Δ32 plus pGADT-7-CCR5, and pGBKT7-CCR5Δ32 plus pGADT7-CXCR4, but not with pGBKT7-CCR5Δ32 plus pGADT7-CXCR2 (under high-stringency conditions), indicating the specificity of the CCR5Δ32 protein interaction with the HIV coreceptors. A strong positive interaction under conditions of medium stringency had a colony count of >1,000. The colonies obtained with medium stringency were further analyzed, and the interaction was confirmed by using a β-Gal colony-lift filter assay (high-stringency conditions). Strongly positive colonies appeared dark blue within 30 min. These results imply that the CCR5Δ32 protein binds directly with CXCR4 and CCR5.

To confirm the yeast two-hybrid system results, we analyzed CCR5Δ32 protein-coreceptor interactions by coimmunoprecipitation. In order to detect CCR5Δ32-coreceptor heterodimers, we used 293-CD4 cells, which constitutively express CXCR4, coinfected with Ad5/Δ32 plus Ad5/CCR5 or Ad5/CCR5 plus Ad5. The cells were first examined for the CCR5Δ32 effect on HIV-1 Env fusion, and as expected, R5 and X4 fusion was dramatically inhibited by CCR5Δ32 protein expression but not by wt CCR5 expression (Fig. 6A). After confirming the

TABLE 2. Interaction of CCR5Δ32 protein with CCR5 and CXCR4

DNA transformed (0.1 μg each)	Interaction under conditions indicated[a]	
	Medium stringency	High stringency
pGBKT7-Lam plus pGADT7-T (negative control)	−	−
pGBKT-7-53 plus pGADT-7-T-Ag (positive control)	++++++	+++
pGBKT-7 plus pGADT-7-CXCR4	−	−
pGBKT-7 plus pGADT-7-CCR5	−	−
pGBKT-7-CCR5Δ32 plus pGADT-7	−	−
pGBKT-7-CCR5Δ32 plus pGADT-7-MVHA	−	−
pGBKT-7-CCR5Δ32 plus pGADT-7-CCR5	+++++	++
pGBKT-7-CCR5Δ32 plus pGADT-7-CXCR4	+++	+
pGBKT-7-CCR5Δ32 plus pGADT-7-CXCR2	±	−

[a] A strong positive interaction (++++++ or +++++) at medium stringency had a colony count of >1,000, whereas +++ indicates colony counts of >500. The ± plate had 25 colonies total, with 24 appearing on one plate and only 1 appearing on the duplicate plate. The colonies obtained at medium stringency were further analyzed, and interactions were confirmed by using a β-Gal colony-lift filter assay (high stringency). Strongly positive colonies (+++) appeared dark blue within 30 min. ++ and +, colonies turned dark blue within 1 and 1.5 h, respectively. CXCR2 is the IL-8 receptor that shares 35% amino acid homology with CXCR4.

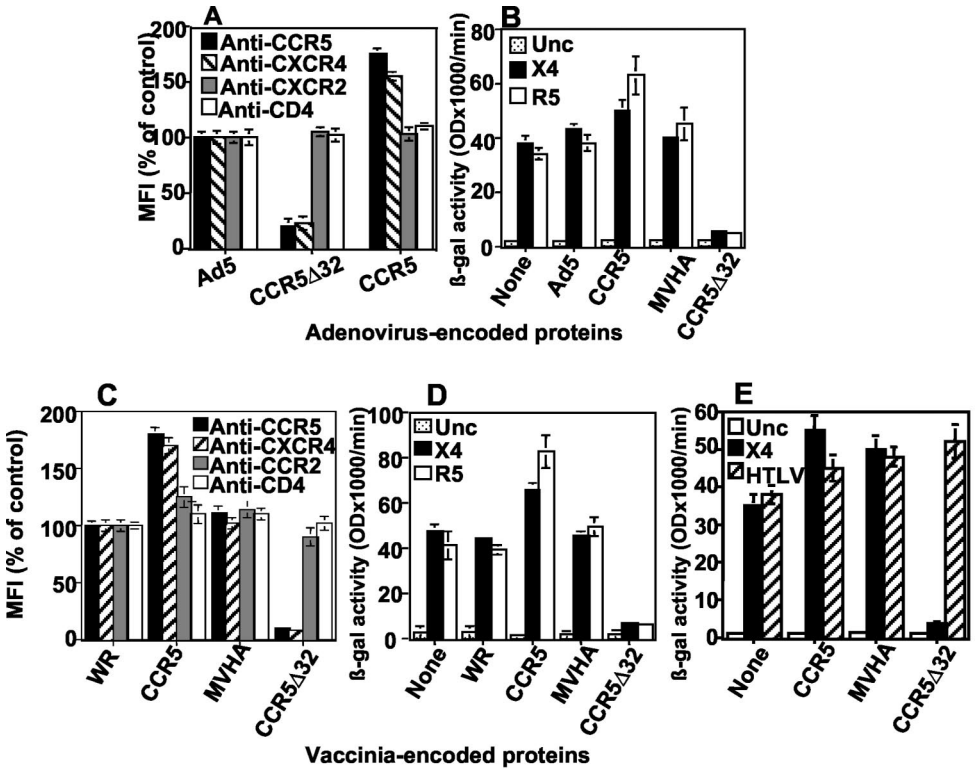

FIG. 5. Exogenous CCR5Δ32 specifically inhibits endogenous CCR5 and CXCR4 expression and HIV coreceptor activity in primary $CD4^+$ cells. (A) Purified $CD4^+$ cells were infected with Ad5 vector, Ad5/Δ32, or Ad5/CCR5 and then stained for cell surface CCR5, CXCR4, CXCR2, or CD4. Staining of cells infected with Ad5 was considered 100% staining. The matched isotype staining was calculated to be <3%. (B) Purified $CD4^+$ cells were infected with Ad5 vectors encoding the proteins indicated on the *x* axis, challenged with HeLa cells expressing either the control Unc, LAV (X4), or Ba-L (R5) HIV-1 Env, and examined for the extent of cell fusion by measuring β-Gal production. (C) Purified $CD4^+$ cells were infected with WR (a vaccinia virus vector control that does not encode any foreign protein) or recombinant vaccinia viruses encoding the proteins indicated on the *x* axis and then were stained for cell surface CCR5, CXCR4, CCR2, or CD4. (D) Separate samples of $CD4^+$ cells were infected with recombinant vaccinia viruses encoding the proteins indicated on the *x* axis, challenged with HeLa cells expressing the same HIV-1 Env proteins as in panel B, and examined for β-Gal production. (E) Some targets used in panel C were challenged with HeLa cells expressing the human T-cell leukemia virus type 1 Env, and the extent of cell fusion was measured similarly. In all of these experiments, fusion with Unc Env represents the background signal resulting from nonspecific cell fusion. The error bars represent replicates of the same experiment. The results shown represent 1 of 10 independent experiments using 10 different donors. MFI, mean fluorescence intensity.

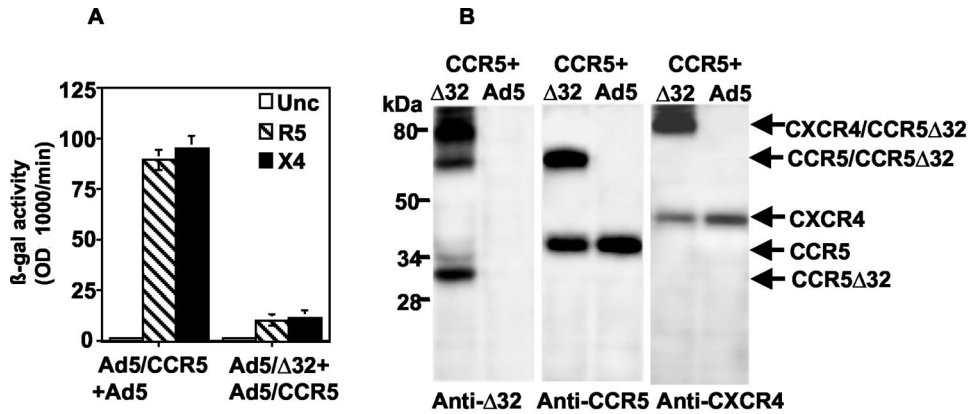

FIG. 6. Heterodimerization of CCR5Δ32 protein with CCR5 and CXCR4. (A) 293-CD4 cells were coinfected with either Ad5/CCR5 plus Ad5 or Ad5CCR5 plus Ad5/Δ32 and challenged with HeLa cells expressing the indicated HIV-1 Envs. The error bars represent replicates of the same experiment. The results shown represent one of three independent experiments. (B) Coimmunoprecipitation analysis. 293-CD4 cells were infected with adenovirus encoding CCR5 (CCR5) plus adenovirus encoding CCR5Δ32 (Δ32) or control adenovirus (Ad5), as indicated at the top of each pair of lanes. Coinfected cells were lysed and immunoprecipitated with anti-CCR5Δ32 antibodies (anti-Δ32). Immunoprecipitates were resuspended in native PFO loading sample buffer and subjected to PFO–10% PAGE and sequential immunoblot analysis with antibodies specific for CCR5Δ32, CCR5, and CXCR4, as indicated at the bottom of each pair of lanes. wt CCR5 was detected with CTC-6 MAbs that do not react with CCR5Δ32 protein. The numbers on the left indicate the positions of molecular weight markers. kDa, kilodaltons. The identity of each band is indicated to the right.

CCR5Δ32 effect in our fusion assay, cell lysates were prepared and immunoprecipitated with anti-CCR5Δ32 antibodies and subjected to PFO-PAGE and blotting. Sequential probing with anti-CCR5Δ32, stripping and reprobing with anti-CCR5 (CTC-6; Protein Design Labs), and stripping and reprobing with anti-CXCR4 antibodies revealed the specific detection of high-molecular-weight bands that do not correspond to the sizes of CCR5Δ32, CCR5, and CXCR4 monomer bands (Fig. 6B). Detection of the same high-molecular-weight protein bands with different antibodies against CCR5Δ32, CCR5, and CXCR4 proteins suggested that complex formation occurred between CCR5Δ32 and either CCR5 or CXCR4. The results provide biochemical evidence for the interaction of the CCR5Δ32 protein with CCR5 and CXCR4.

Expression of CCR5Δ32 protein confers resistance to diverse HIV-1 strains. To show efficient gene delivery by our adenovirus vector system, we used a similar recombinant adenovirus, Ad5/GFP, encoding GFP under the control of a cytomegalovirus promoter. The infection of primary $CD4^+$ cells at 25 to 50 PFU/cell resulted in efficient GFP expression (green fluorescence) in most of the infected $CD4^+$ cells (data not shown). This high efficiency of gene delivery allowed a detailed analysis of the biological activities of the CCR5Δ32 protein in primary cells. In order to examine Δ32 effects on HIV-1 infection, healthy seronegative (+/+) $CD4^+$ cells were transduced to express Ad5-encoded CCR5 or Ad5-encoded CCR5Δ32 protein. The expression of CCR5Δ32 protein in this experiment was verified by Western blotting to show that the Ad5-encoded CCR5Δ32 protein was expressed at levels comparable to those endogenously made in −/− $CD4^+$ cells (Fig. 7A). Purified $CD4^+$ cells expressing wt CCR5 protein were sensitive to HIV-1 infection and showed the expected levels of p24 protein. In contrast, $CD4^+$ cells expressing Ad5-encoded CCR5Δ32 protein showed a dramatic inhibition of productive infection by X4 (Fig. 7B), R5 (Fig. 7C), and R5X4 isolates (Fig. 7D). Since primary +/+ cells express endogenous CCR5, the expression of Ad5-encoded CCR5 was verified by FACS analysis of infected cells. The results indicated a twofold augmentation of CCR5 surface expression in Ad5/CCR5-infected cells (data not shown). These results are consistent with the CCR5Δ32 effects on Env fusion and provide evidence for a broad protective CCR5Δ32 effect in primary cells.

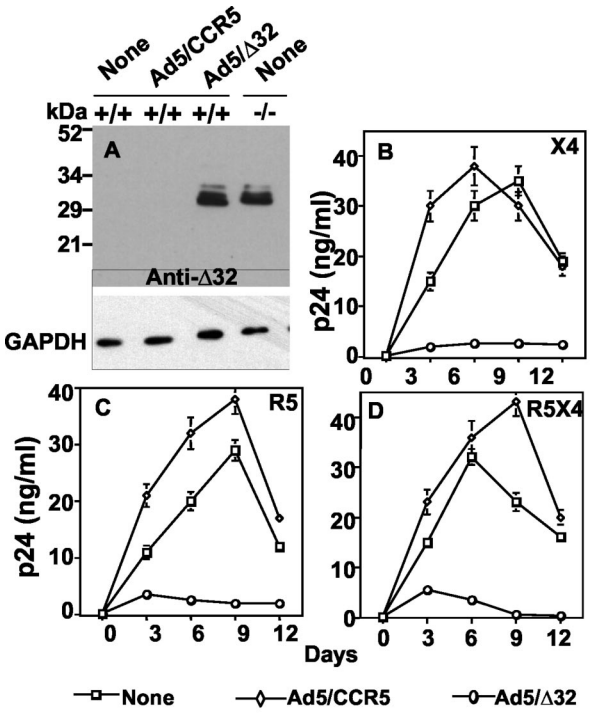

FIG. 7. Gene delivery of CCR5Δ32 into $CD4^+$ cells from HIV-seronegative CCR5 (+/+) individuals confers resistance to R5 and X4 HIV-1 infection. (A) Expression of CCR5Δ32 protein in $CD4^+$ cells. Lysates of cells from individuals homozygous for wt CCR5 (+/+) or CCR5Δ32 (−/−) that had been infected with no virus (none) or adenovirus encoding CCR5 (Ad/CCR5) or CCR5Δ32 (Ad5/Δ32) were analyzed by Western blotting using anti-CCR5Δ32-specific antiserum (anti-Δ32). Note that the levels of exogenous and endogenous CCR5Δ32 protein were similar in appropriate samples. GAPDH was monitored to assess equivalent loading of samples. The numbers at the left indicate the positions of protein standards. kDa, kilodaltons. (B to D) HIV replication kinetics in $CD4^+$ cells. Control cells were incubated with no virus (none; open squares) and compared to cells infected with either Ad5/Δ32 (open circles) or Ad5/CCR5 (open triangles) at 50 PFU/cell for each virus with regard to productive infection by the X4 HIV-1 strain IIIB (B), the R5 strain Ba-L (C), and the R5X4 strain 89.6 (D). The amount of p24 antigen in the cell-containing supernatants was measured over time by enzyme-linked immunosorbent assay. A zidovudine control infection resulted in p24 values below 1 ng/ml (not shown). The results shown are the means ± standard errors of the means from one experiment representative of five independent experiments using five different donors.

DISCUSSION

Our data demonstrate that the truncated mutant protein encoded by *CCR5Δ32* is endogenously expressed in PBMCs isolated from *CCR5Δ32* homozygotes and is biologically active in primary $CD4^+$ cells. In particular, the protein exerts a dominant-negative effect on the expression of wt CCR5 and CXCR4, with the consequence that cells expressing it become less susceptible to infection with prototypic R5, R5X4, and X4 strains of HIV. The dominant-negative mechanism appears to involve direct binding of CCR5Δ32 to wt CCR5 and CXCR4. Taken together, our data suggest that *CCR5Δ32* may genetically restrict HIV pathogenesis in at least two ways, (i) by reducing in heterozygotes or preventing in homozygotes the production of normal CCR5 and (ii) by encoding a biologically active mutant protein that is able to scavenge normal CCR5 and CXCR4.

This is the first study to test the biological effects of recombinant CCR5Δ32 protein in primary $CD4^+$ cells. The finding that CXCR4 expression is downregulated by CCR5Δ32 protein activity is relevant to disease progression and pathogenesis since CXCR4-using viruses emerge at the late symptomatic stage of AIDS. The novelty of our approach is the use of an efficient vector system that delivers the CCR5Δ32 gene to all cells under analysis. The observed downmodulation of CXCR4 is unlikely to be due to an artifact of massive, unregulated overexpression of the CCR5Δ32 protein, since firstly, this vector system is replication defective in primary cells, and secondly, our analysis indicated that the levels of CCR5Δ32

protein driven by our vector were comparable to those endogenously made in −/− $CD4^+$ cells.

The dominant-negative effect we describe can only occur in cells that coexpress CCR5 and CXCR4. Previous studies have demonstrated that CCR5 and CXCR4 are expressed predominantly on $CD4^+$ $CD45RO^+$ and $CD4^+$ $CD45RA^+$ cells, respectively, and that CXCR4 is widely expressed on PBMCs, whereas CCR5 is more restricted, suggesting that the CCR5Δ32 protein would be expressed in only a small proportion of $CXCR4^+$ cells (11, 24). The results, however, depended on the analysis of a limited number of donors. We have found that donor variability and the method of stimulation are important factors that influence the abundance of $CXCR4^+$ $CCR5^+$ double-positive cells. Our study considered both CD3- and CD4-gated cells to show that different donors responded differently to cell activation, leading to dramatic differences in the percentages of double-positive cells. Donor variability in coreceptor expression has also been demonstrated in unstimulated PBMCs (23, 24). We consistently found that the CD4-gated cell population expressed a significantly higher percentage of double-positive cells.

Identifying changes in CXCR4 levels in cells that coexpress CCR5 may be difficult since CXCR4 is widely expressed on human lymphocytes. A comparative analysis of CXCR4 expression in a significant number of unstimulated $CD4^+$ cells isolated from −/− and +/+ individuals has not previously been reported. Previous studies that performed CXCR4 staining in −/− cells used total PBMCs from either one (46) or two (45) individuals. Although −/− PBMCs express CXCR4, the level of expression may vary with cell activation, and this may affect the degree to which the cells support X4 HIV replication. In particular, we have shown that cell activation causes up-regulation of CXCR4 and reciprocal down-regulation of CCR5Δ32 protein in −/− $CD4^+$ T cells. Down-regulation of CCR5 and up-regulation of CXCR4 in wt CCR5 PBMCs have previously been reported to occur as a result of cell activation (11).

Previous studies reported different results in terms of HIV-1 X4 infection of −/− PBMCs. For example, reduced CXCR4 expression (compared to healthy wt CCR5 individuals) has been reported for two different −/− individuals and one +/− individual (45). The lower level of CXCR4 staining correlated with resistance to X4 infection by the two −/− PBMC samples (45). The same −/− PBMC samples (45) were shown to be partially permissive for X4 infection upon challenge with a high-input X4 virus (15). Although CXCR4 staining has not been compared in $CD4^+$ cells from −/− individuals, the literature contains a significant number of studies showing the resistance of −/− PBMCs to X4 infection (15, 33, 36, 40, 46). Other studies reported lower X4 infectivity in PBMCs isolated from two exposed, uninfected (−/− and +/−) individuals than in those from two unexposed, uninfected (+/+ and +/−) individuals (45). Additionally, Salkowitz et al. (37) reported that the concentration of PHA used for PBMC activation is critical for the observation of resistance to the X4 NL4-3 isolate. Taken together, these studies indicated that individual variability, virus input, and the method of cell activation have significantly contributed to the previously reported results regarding X4 infection of −/− PBMCs.

None of the previous studies that analyzed the mechanism of the Δ32 effects had utilized primary $CD4^+$ cells as targets for CCR5Δ32 protein expression (8, 13, 42). Those studies utilized different cell lines in their analyses of the TDN effect and reported opposite results (8, 13, 42). While Benkirane et al. (8) and Chelli et al. (13) reported a partial TDN effect for the mutant CCR5Δ32 protein against R5 infection and/or fusion, Venkatesan et al. (42) reported no such effect. We believe that these discrepant findings were probably a consequence of variable transfection efficiencies that resulted in a large number of cells lacking expression of mutant CCR5Δ32 proteins but expressing abundant levels of coreceptors. Therefore, examining the inhibitory effect of CCR5Δ32 under these conditions may not reveal the actual activity of the mutant protein. The two studies that reported CCR5Δ32 protein activity against R5 did not detect the TDN effect against X4 (8, 13). The reasons for this could be poor expression of the mutant protein by transfection and the use of cell lines that express endogenous CXCR4. Low levels of exogenous mutant protein expression may be insufficient to induce a detectable TDN effect on endogenous CXCR4.

All G-protein-coupled receptors (GPCRs) are predicted to share a similar molecular structure characterized by the presence of seven transmembrane helices connected by three intracellular and three extracellular loops (9). Functionally defective, naturally occurring mutations in GPCRs, including CCR5Δ32, have been reported to cause impaired processing and intracellular retention. Previous studies on members of this family have suggested that 7TM receptors in some cases may exist in oligomeric forms (14, 20, 32, 44). Zhu et al. have demonstrated that the truncated mutant vasopressin receptors are able to form heterodimers with the full-length receptor and that this complex formation inhibited wt receptor function (49). The same study found that truncated vasopressin receptor mutants specifically inhibited the function and cell surface trafficking of the coexpressed full-length vasopressin receptor but had no effect on the function of other GPCRs. An analysis of the domains of CCR5 and CXCR4 that interact with the mutant protein will probably lead to the identification of common epitopes that will have implications for the design of drugs that down-regulate the major HIV coreceptors.

Our study suggests that CXCR4 expression is limiting for viral entry (Fig. 5) and that there may be an optimum number and density of coreceptor molecules for HIV-1 infection, which is in agreement with studies from Kabat's group (22). By extension, the CCR5Δ32/CXCR4 and CCR5Δ32/CCR5 ratios may be critical determinants for the inhibition of X4 and R5 HIV infection, respectively, by the mutant protein. Previous studies have reported that protection against HIV-1 in heterozygotes might depend on the ratio of wt to mutant CCR5 mRNA (31). However, detailed analysis of the CCR5Δ32/CXCR4 and CCR5Δ32/CCR5 protein ratios in these individuals will be necessary to confirm such findings.

Despite the absence of CCR5 in −/− individuals, CXCR4 is rarely used as an alternate coreceptor to infect $CD4^+$ cells. Macrophages have classically been regarded as relatively resistant to X4; however, some primary X4 isolates are able to infect macrophages via CXCR4 (38, 43). Recently, Naif et al. described an R5X4 isolate capable of infecting −/− macrophages through CXCR4 (28). Furthermore, a number of primary syncytium-inducing HIV-1 strains were found to be of the

R5X4 type, which can utilize both CCR5 and CXCR4 and have the ability to infect primary macrophages in vitro (7, 39, 47). Our results indicate that two of these infected −/− individuals lacked expression of the CCR5Δ32 protein. We hypothesize that the absence of detectable CCR5Δ32 protein in infected −/− individuals resulted in the loss of the TDN effect against CXCR4, leading to X4 infection. The lack of CCR5Δ32 protein expression may be explained by a defect either in transcription or translation. These possibilities are currently under investigation.

In summary, this study provides the first evidence that expression of recombinant CCR5Δ32 protein in human PBMCs confers broad protection against R5 and X4 strains of HIV-1. We have shown that the truncated CCR5Δ32 protein can act as a negative regulator of wt CCR5 and CXCR4. The dominant-negative activity of the mutant CCR5Δ32 protein correlated with its ability to reduce the cell surface expression of the major HIV coreceptors and to form heterodimeric complexes with CCR5 and CXCR4. Understanding the molecular nature of the protective effects of the CCR5Δ32 protein may lead to new insights into the interrelationships of these molecules that are employed by HIV-1 and perhaps to the development of novel therapeutic agents capable of conferring broad antiviral effects. New insights gained in the mechanism of action of this naturally occurring protein may be incorporated into new approaches to induce resistance to HIV-1.

ACKNOWLEDGMENTS

We thank Hal Broxmeyer, S. Spinola, and A. Srivastava for their comments on the manuscript, Hassan Naif, Haynes Sheppard, and Susan Buchbinder for providing PBMC samples from infected −/− individuals, and Frank Graham for providing Ad5Pol3. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (i) recombinant human IL-2 from Maurice Gately, Hoffmann-La Roche, Inc.; (ii) HIV-1 89.6 from Ronald Collman; (iii) HIV-1 Ba-L from Suzanne Gartner, Mikulas Popovic, and Robert Gallo; and (iv) HIV-1 IIIB from Robert Gallo.

This study was supported by NIH grant A152019-01A1 to G.A. PBMC samples were obtained under Indiana University IRB study 9812-05.

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